ORIGINAL ARTICLE

Alterations in serum levels of selected markers of oxidative imbalance in adult celiac patients with extraintestinal manifestations: a pilot study

Agnieszka Piątek-Guziewicz[1], Paweł Zagrodzki[2,3], Paweł Paśko[3], Mirosław Krośniak[3], Agata Ptak-Belowska[4], Magdalena Przybylska-Feluś[5], Tomasz Mach[5], Małgorzata Zwolińska-Wcisło[5]

1 Department of Gastroenterology and Hepatology, The University Hospital, Kraków, Poland
2 The Henryk Niewodniczański Institute of Nuclear Physics, Kraków, Poland
3 Department of Food Chemistry and Nutrition, Jagiellonian University Medical College, Kraków, Poland
4 Department of Physiology, Jagiellonian University Medical College, Kraków, Poland
5 Unit for Clinical Dietetics, Department of Gastroenterology, Hepatology and Infectious Diseases, Jagiellonian University Medical College, Kraków, Poland

KEY WORDS

celiac disease, glutathione peroxidase, nitric oxide, oxidative stress, vitamin E

ABSTRACT

INTRODUCTION Oxidative stress is considered to be one of the mechanisms responsible for gluten toxicity, but its role in celiac disease (CD) remains unclear.

OBJECTIVES The aim of the study was to evaluate oxidative imbalance in the pathomechanism of CD by determining the concentrations of nitric oxide (NO) and selected antioxidant parameters.

PATIENTS AND METHODS The study involved 197 adult patients: 53 patients with untreated active CD, 92 celiac patients on gluten-free diet (GFD), and 52 controls. The serum levels of antioxidants (uric acid, bilirubin, ferritin, albumin), celiac antibodies, NO, glutathione peroxidase 3 (GPx3), and vitamin E were measured. A histopathological study of duodenal biopsy was performed.

RESULTS Celiac patients had higher uric acid concentrations than controls ($P < 0.001$). NO levels were higher in patients with active CD than in controls ($P < 0.01$) and were correlated with the degree of mucosal damage ($r^2 = 0.04$; $P = 0.01$). Vitamin E levels were decreased in celiac patients ($P < 0.01$), and GPx3 activity was reduced in patients with active CD compared with controls ($P < 0.001$).

CONCLUSIONS Oxidative imbalance may be involved in the pathomechanism of CD in adults. GFD only partially reduces oxidative stress. Serum NO levels seem to be a marker of the effectiveness of treatment. Uric acid may act as an antioxidant in CD.

Correspondence to:
Małgorzata Zwolińska-Wcisło, MD, PhD, Zakład Dietetyki Klinicznej, Katedra Gastroenterologii, Hepatologii i Chorób Zakaźnych, Uniwersytet Jagielloński, Collegium Medicum, ul. Śniadeckich 5, 31-531 Kraków, Poland, phone: +48 12 424 73 40, e-mail: mzwcislo@su.krakow.pl
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INTRODUCTION Celiac disease (CD) is a common heritable chronic condition, in which the ingestion of the gluten fraction of wheat or the adequate proteins from rye and barley causes chronic inflammation of the small intestine.[1] The clinical presentation of CD is diversified and varies with the age of patients, duration of illness, and severity of the disease, and extraintestinal manifestations may be present.[2] The classic presentation of CD as a predominately pediatric disease is characterized by symptoms such as chronic diarrhea, bloating, and growth failure. Adult celiac patients have presented with different clinical features, including the nonclassic, subclinical, or asymptomatic form of the disease.

The pathogenesis of CD is complicated and still not fully explained. Besides genetic predisposition, the immunological mechanism plays the main role in the disease development. Recent studies have indicated the direct cytotoxic effect of gluten on enterocytes.[3] It has been assumed that oxidative stress (OS), because of an increase in the concentration of reactive oxygen species (ROS) and a decrease of antioxidant capacity, is

TABLE 1 Characteristics of the study groups

Patient groups	Active CD (n = 53)	Treated CD[a] (n = 92)	Controls (n = 52)
Age, y, mean (SD)	35.9 (11.7)	42.6 (15.1)	39.6 (12.7)
Sex, female/male, n	44/9	77/15	44/8

a Celiac patients on a gluten-free diet

Abbreviations: CD, celiac disease

one of the processes possibly involved in gliadin toxicity.[1] Toxic oligopeptides collected in the small intestine may lead to toxic effects in genetically susceptible individuals.[4] However, although OS is considered to be one of the mechanisms responsible for gluten (gliadin) toxicity, its role in patients with CD has not been fully explored. Oxidative imbalance induced by gliadin peptides in enterocytes leads to the activation of the transcription of proinflammatory cytokines and enzymes such as inducible nitric oxide synthase (iNOS), which in turn leads to increased production of nitric oxide (NO) metabolites promoting OS.[5] NO takes part in the pathogenesis of various inflammatory disorders, such as Crohn disease, ulcerative colitis, and in certain studies, higher NO levels in patients with inflammatory bowel disease have been shown.[6,7] Some studies have reported that reactive nitrogen species also take part in the pathogenesis of CD. The constitutive enzyme iNOS is expressed in human enterocytes, with increased activity in patients with untreated CD and with partial correction in celiac patients on gluten-free diet (GFD).[8,9] It is possible that increased concentration of fasting plasma NO is a consequence of increased iNOS expression in the small intestine. Excessive production of NO may lead to increasing mucosal permeability due to damage to gut barrier function.[10]

In normal conditions, the harmful effects of ROS are opposed by the antioxidant defense system consisting of antioxidant enzymes (glutathione peroxidase [GPx], glutathione reductase [GR], superoxide dismutase [SOD], and catalase), nonenzymatic antioxidants (such as glutathione [GSH], albumin, bilirubin, ceruloplasmin, and uric acid [UA]), as well as nutritional antioxidants (carotenoids and vitamins A, C, and E).[11] The reduced antioxidant defenses may make the inflamed mucosa more sensitive to oxidative tissue damage and may disrupt the recovery and integrity of the mucosa.

Using thiobarbituric acid-reactive substances as a marker of OS, Odetti et al[12] showed that redox equilibrium is impaired in patients with CD. They also observed decreased serum α-tocopherol levels in a group with silent CD in comparison with controls. Earlier studies also showed that the activity of SOD is markedly increased in pediatric patients with CD, while the activity of GPx is significantly decreased.[13] Interestingly, studies concerning the activity of GPx, as most studies on the role of OS in CD, have been conducted so far only in children with classic clinical symptoms of malabsorption syndrome and villous atrophy. Studies on oxidative imbalance in adult celiac patients with extraintestinal manifestations are sparse. To the best of our knowledge, there is lack of research on naturally occurring nonenzymatic antioxidants and their role in CD. Therefore, we aimed to evaluate the involvement of OS in the pathomechanism of CD and to monitor antioxidant defense in adult celiac patients with extraintestinal manifestations. For that purpose, we examined the levels of fasting plasma nitrate as a marker of endogenous NO production and monitored individual components of antioxidant capacity: GPx3 activity and serum levels of UA, ferritin, albumin, bilirubin, and vitamins D and E.

PATIENTS AND METHODS The study included 197 outpatients and inpatients of the Department of Gastroenterology and Hepatology of the University Hospital in Kraków, Poland (TABLE 1). The first group comprised 53 patients with active CD (newly diagnosed patients as well as patients with CD not adhering to GFD, with positive celiac antibody titers). The second group included 92 patients with treated CD who were on GFD for at least 2 years (mean [SD] disease duration, 10.4 [8.1] years). The third group included 52 patients with functional disorders of the gastrointestinal tract, without abnormalities on upper gastrointestinal endoscopy or on serological and histological examinations, who served as controls.

The diagnosis of CD was based on clinical symptoms, positive celiac antibody titers (antitissue transglutaminase antibodies [TGA] and/or antiendomysial antibodies [EmA]) and the characteristic histologic features of small intestinal biopsies.

Celiac patients showed nonclassic signs and symptoms including extraintestinal manifestations such as anemia, iron deficiency without gastrointestinal symptoms, chronic abdominal pain without typical malabsorption syndrome, osteoporosis, osteopenia, as well as asymptomatic disease. Patients with Duhring disease, diabetes, inflammatory bowel disease, current infectious disease, history of cancer, chronic hepatobiliary disease, chronic renal impairment, alcohol abuse, or those receiving chronic nonsteroidal anti-inflammatory drugs, antioxidant supplements, oral contraceptives, or immunosuppressive and immunostimulatory drugs were excluded from the study. All patients were nonsmokers.

All participants underwent upper gastrointestinal endoscopy, and at least 4 duodenal specimens were obtained for a microscopic examination. The degree of intestinal mucosal damage was then classified in accordance with the Marsh classification.[14]

On the day of gastroscopy, blood was collected by venipuncture to assess serum levels of TGA and/or EmA, NO, and antioxidants: vitamins D and E, GPx3, UA, albumin, ferritin, and bilirubin. The TGA titer was evaluated using a commercial enzyme-linked immunosorbent assay (ELISA) kit

(Aesku Diagnostics GmbH, Wendelsheim, Germany). A concentration higher than 15 U/ml was considered positive. The EmA titer was assessed with the immunofluorescence method. A titer higher than 1:10 was regarded as positive.

All study participants provided written informed consent to participate in the study. The study protocol was approved by the Ethical Committee of Jagiellonian University Medical College (No. KBET/174/B/2013) and conducted according to the Declaration of Helsinki.

Blood sample collection Venous blood samples were obtained in the fasting state. The levels of albumin, UA, ferritin, bilirubin, and vitamin D were evaluated on the same day. For GPx3, NO, and vitamin E assays, blood samples were centrifuged at 1000 *g* for 15 minutes at a temperature of 4°C, and the serum was collected and stored at a temperature of –80° C until further assay.

Nitric oxide, vitamin E, and glutathione peroxidase-3 assays The levels of NO and vitamin E were evaluated using commercially available ELISA kits according to the manufacturer's protocol. NO levels were determined with the Parameter Total Nitric Oxide and Nitrate/Nitrite KGE001 kits (R&D Systems, Minneapolis, Minesotta, United States) and vitamin E levels—with General Vitamin E Elisa Kit E0922Ge (EIAab Science, Wuhan, China). A spectrophotometric microplate reader (Stat Fax 2100 Awareness Technology Inc., Palm City, Florida, United States) was used to determine the optical density at 540 nm and 450 nm, respectively. The levels of NO and vitamin E were calculated from a standard curve. The nitrite concentration in the sample was determined by subtracting the endogenous nitrite concentration from the total nitrite concentration. The plasma GPx3 level was evaluated with hydrogen peroxide as the substrate, as described previously.[15]

Statistical analysis For all parameters, descriptive statistics were calculated. The normality of the distribution of parameters was checked by the Kolmogorov–Smirnov test. Comparisons between the patient groups were performed using either the analysis of variance with the Tukey post hoc test for parameters with normal distribution and homogenous variances or the Kruskal–Wallis test with the Dunn post hoc test for all other parameters. Differences with a *P* value of less than 0.05 were considered significant. Statistical calculations were done using the commercially available packages STATISTICA PL v.10 (StatSoft, Tulsa, Oklahoma, United States) and GraphPad Prism v.3.02 (GraphPad Software, San Diego, California, United States), while the correlation weights were calculated using the software delivered by MP System Sp. z o.o. (Chrzanów, Poland).

RESULTS

Blood tests in controls and celiac patients The mean ferritin level was lower in patients with untreated active CD than in controls ($P < 0.001$). The median ferritin level in patients with untreated CD was lower than that in treated patients ($P < 0.01$).

Serum albumin levels did not differ between the celiac groups (mean [SD], 43.0 [4.3] g/l in patients with untreated active CD and 43.9 [3.0] g/l in patients with treated CD), while they were lower in the control group ($P < 0.01$).

Serum UA concentrations were elevated only in celiac patients: in 4 patients (7.5%) with active CD and in 4 patients (4.3%) on GFD. UA levels were higher in the celiac groups than in controls ($P < 0.001$), while bilirubin levels were lower in patients with active CD than in controls ($P < 0.05$).

Reduced vitamin D levels were reported in 37 patients (69.8%) with active CD, in 61 patients (66.3%) with treated CD, and in 18 controls (34.6%). Moderate vitamin D deficiency (10–19 ng/ml) was reported in 24 patients (45.3%) with untreated CD and 21 patients (22.8%) with treated CD; severe deficiency (<10 ng/ml) was reported in 8 celiac patients (5.5%) and in none of the controls. The mean vitamin D level was lower in patients with active CD than in controls or treated celiac patients ($P < 0.001$ and $P < 0.05$, respectively), and was lower in treated celiac patients than in controls ($P < 0.05$).

The results of biochemical tests are presented in TABLE 2.

Serum nitric oxide levels The serum NO level was higher in patients with active CD compared with controls (mean [SD], 86.4 [61.4] µmol/l vs 56.8 [37.3] µmol/l, $P < 0.01$). The mean serum NO level was lower in patients on GFD than in untreated celiac patients and higher than in controls, but the differences were not significant (TABLE 2 and FIGURE 1).

The degree of intestinal mucosal damage correlated with serum NO levels in celiac patients ($r^2 = 0.04$; $P = 0.01$).

Serum vitamin E levels Serum vitamin E levels were lower in untreated celiac patients and in treated celiac patients than in controls (mean [SD], 41.1 [36.8] µmol/l vs 48.1 [20.8] µmol/l and 37.3 [32.1] µmol/l vs 48.1 [20.8] µmol/l, respectively, $P < 0.01$).

Vitamin E deficiency, defined as the levels lower than 16.2 µmol/l,[20] was detected in over 60% of celiac patients and in 3.7% of controls. Optimal vitamin E levels (>30 µmol/l) required for protection against cardiovascular disease and cancer were reported in less than 40% of celiac patients and in more than 96% of controls (TABLE 2 and FIGURE 2).

Serum glutathione peroxidase-3 levels We observed decreased activity of GPx3 in celiac groups compared with controls. The difference was significant between active celiac patients and controls (mean [SD], 414.7 [107.2] U/l vs 485.4 [89.1] U/l, $P < 0.001$). Treated celiac patients showed higher activity of GPx3 compared with untreated celiac

TABLE 2 Results of blood tests in the study groups (only the tests with statistically significant results are presented)

Variable	Controls (n = 52)		Celiac patients (n = 145)			
			Treated CD[a] (n = 92)		Active CD (n = 53)	
	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
Albumin, g/l	42.0 (3.8)	41.0	43.9 (3.0)[c]	44.0	43.0 (4.3)	43.0
Bilirubin, µmol/l	10.0 (3.8)	9.4	9.1 (4.4)	8.2	8.5 (5.3)[e]	7.7
Ferritin, µg/l	53.9 (24.5)	53.0	59.5 (81.9)	35.0	52.3 (163.2)	22.0[d,f]
GPx3, U/l	485.4 (89.1)	494.2	453.8 (90.4)	460.1	414.7 (107)[d]	403.2
NO, µmol/l	56.8 (37.3)	45.3	69.7 (41.8)	60.2	86.4 (61.4)[c]	75.8
Uric acid, µmol/l	190.1 (41.8)	189.0	259.4 (49.6)[d]	252.0	261.3 (55.2)[d]	242.0
Total vitamin D, ng/ml	28.1 (5.5)	29.2	24.4 (10.8)[e]	23.5	20.8 (10.3)[b,d]	18.8
Vitamin E, µmol/l	48.1 (20.8)	49.9	37.3 (32.1)[c]	28.9	41.1 (36.8)[c]	30.3

a Celiac patients on a gluten-free diet; **b** $P < 0.05$ vs treated CD; **c** $P < 0.01$ vs controls; **d** $P < 0.001$ vs controls; **e** $P < 0.05$ vs controls; **f** $P < 0.01$ vs treated CD

Abbreviations: GPx3, glutathione peroxidase 3; NO, nitric oxide; others, see **TABLE 1**

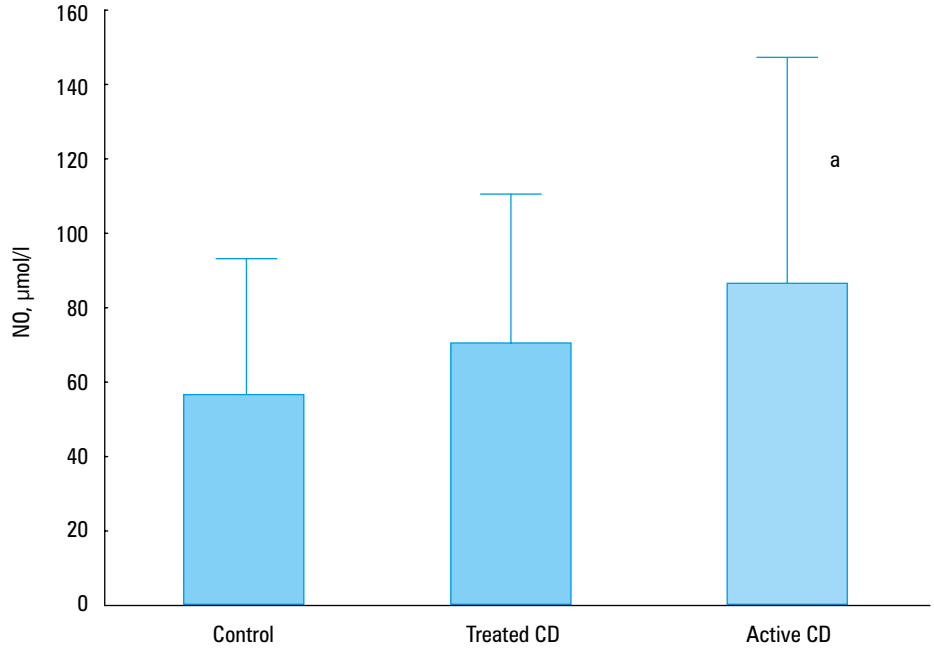

FIGURE 1 Serum concentrations of nitric oxide (NO) in controls, patients on gluten-free diet (treated CD), and patients with the active form of celiac disease
a $P < 0.01$ (compared with the control group)

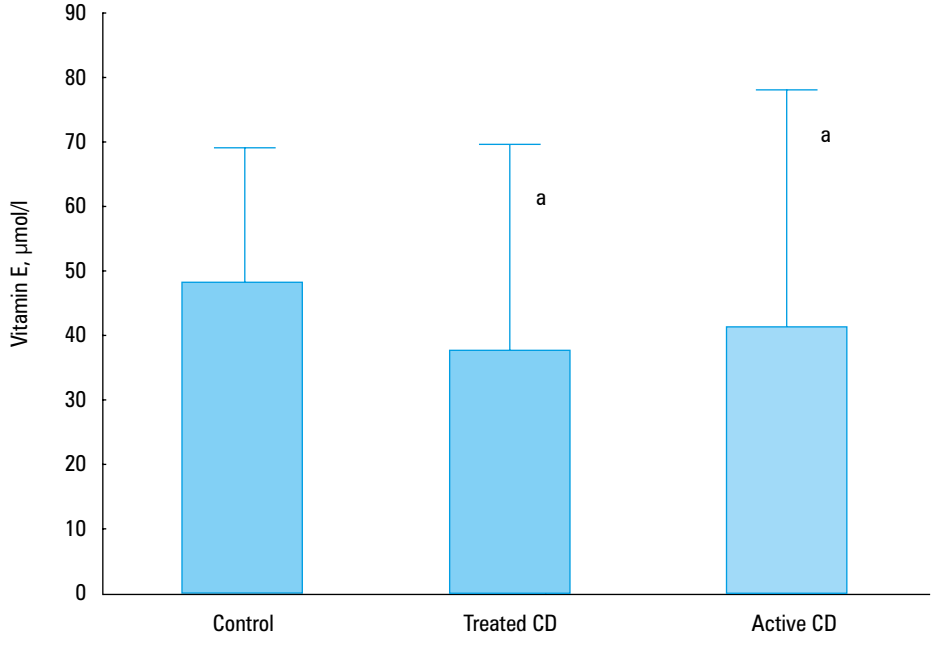

FIGURE 2 Serum concentrations of vitamin E in controls, patients on gluten-free diet (treated CD), and patients with the active form of celiac disease
a $P < 0.01$ (compared with the control group)

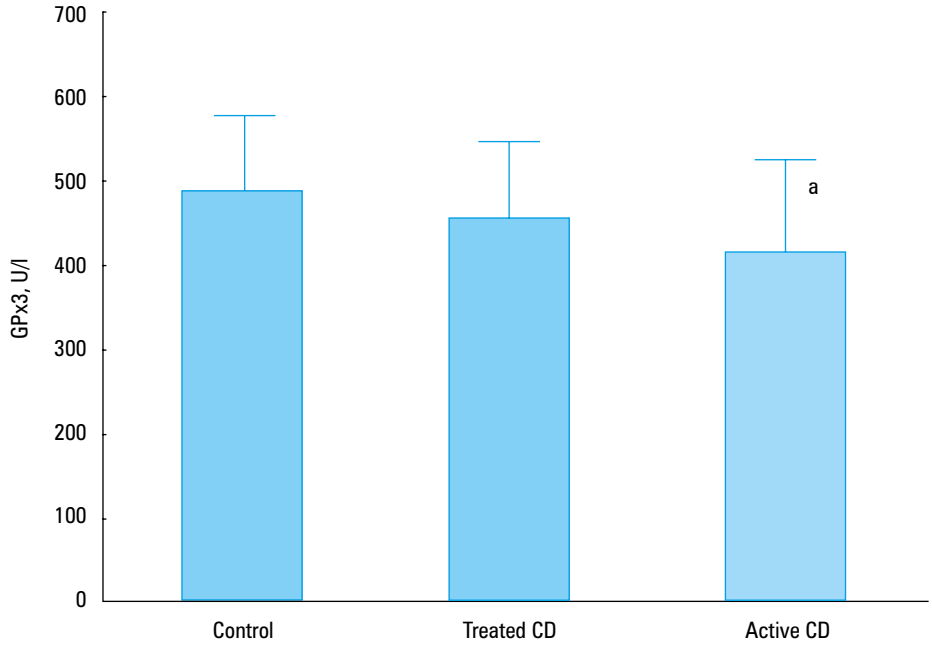

FIGURE 3 Serum concentrations of glutathione peroxidase (GPx3) in controls, patients on gluten-free diet, and patients with the active form of celiac disease

a $P < 0.01$ (compared with the control group)

TABLE 3 Clinical characteristics of the study groups: serum levels of celiac antibodies and the degree of intestinal mucosal damage

		Controls (n = 52)	Treated CD[a] (n = 92)	Active CD (n = 53)
Antibody titer[b]				
0		52 (100)	89 (96.7)	2 (3.8)
1		0	3 (3.3)	14 (26.4)
2		0	0	18 (34)
3		0	0	19 (35.8)
Mean (SD)		0	0.03 (0.2)	2.02 (0.9)[d,e]
Median		0	0	2
Degree of intestinal mucosal damage[c]				
Normal mucosa	0	51 (96)	31 (33.7)	3 (5.7)
Marsh 1	1	1 (3.8)	31 (33.7)	11 (20.8)
Marsh 2	2	0	0	0
Marsh 3a	3	0	14 (15.2)	11 (20.8)
Marsh 3b	4	0	14 (15.2)	15 (28.3)
Marsh 3c	5	0	2 (2.2)	13 (24.5)
Mean (SD)		0.1 (0.2)	1.5 (1.6)	3.2 (1.7)
Median		0.0	1.0[c]	4.0[d,e]

Data are presented as number (percentage) of patients unless indicated otherwise.

a Celiac patients on a gluten-free diet

b Antibody titer: 0, negative; 1, normal (TGA $<3 \times$ ULN; EmA [+]); 2, high ($3 \times$ ULN $<$ TGA $<10 \times$ ULN; EmA [++]); 3, very high (TGA $>10 \times$ ULN; EmA [+++])

c The degree of intestinal mucosa damage was classified in accordance with Marsh parameters, and each stage was given a score from 0 (normal mucosa) to 5 (total villous atrophy); data are presented as number (percentage) of patients.

d $P < 0.001$ vs controls

e $P < 0.001$ vs treated CD

Abbreviations: EmA, antiendomysial antibodies, TGA, antitissue transglutaminase antibodies; ULN, upper limit of normal; others, see TABLE 1

patients, but the difference was not significant (TABLE 2 and FIGURE 3).

Serum levels of celiac antibodies and the degree of intestinal mucosal damage The serum levels of celiac antibodies were negative in controls. Significantly higher levels were observed in untreated celiac patients. In treated celiac patients, the levels of antibodies were significantly lower than in untreated celiac patients. The degree of intestinal damage was the lowest in controls, while it was higher in patients with treated CD and the highest in patients with untreated active CD. The differences between the groups were significant (TABLE 3).

DISCUSSION

The pathogenesis of CD has not been fully explained. Because of an increase of ROS and the reduced antioxidant protection, inflammation and OS seem to participate in the pathomechanisms of the disease. Most studies concerning the pathomechanism of CD involved children with classic clinical symptoms of malabsorption syndrome. However, malabsorption alone does not explain the pathophysiology and clinical course of numerous extraintestinal manifestations and nonclassic symptoms that predominate in adult patients with CD. Other possible mechanisms include gluten toxicity with oxidative imbalance and autoimmunity.

To the best of our knowledge, our study is the first to have analyzed the serum levels of the parameters of oxidative imbalance in adult celiac patients with extraintestinal manifestations.

NO is produced by NO synthase, an enzyme found in a number of cell types. NO has different functions in the gastrointestinal tract, both physiological and pathological, and NO synthase has 2 forms: constitutive and inducible.[16] The latter produces NO in response to pathological impulses such as inflammatory process.[17] Some authors noted increased levels of NO in the serum

and urine of children with CD, with a positive correlation between the concentration of NO and increased concentration of iNOS in the small intestine.[10,18-20] Murray et al[17] showed higher plasma NO concentrations in adult patients with CD than in treated patients with CD on GFD and those with other upper gastrointestinal disorders. Ertekin et al[19] reported that serum NO levels decreased after a 1-year GFD in children with CD, and there was a significant correlation between the degree of intestinal mucosal damage and serum NO levels. Higher production of NO metabolites, and, in consequence, nitrosative stress, promote the impairment of tight junctions in the small intestine of CD patients, perhaps by downregulating the expression of zonula occludens-1.[21] These observations are in line with the results of our study. We also reported a significant correlation between the degree of intestinal mucosal damage and the serum NO level. Spencer et al[22] showed that plasma NO levels decreased just after the introduction of GFD in adults with CD and correlated with intestinal changes at diagnosis but not after 6 months of treatment with GFD. All these results may imply that oxidative injury induced by NO does not depend on the clinical form but is associated with histologic changes. Increased serum levels of NO are probably a marker of an ongoing inflammation in the small intestine in untreated adult celiac patients in the same way as in untreated children with CD with classic manifestations. The persistent significant elevation of serum NO levels despite dietary compliance may suggest refractory CD and indicate the need for further study, including control endoscopy. Hence, it seems to be a marker of the effectiveness of treatment.

Elevated OS with increased serum levels of NO has also been observed in neurodevelopmental conditions such as attention-deficit hyperactivity disorder and autism spectrum disorders.[23] Although the relationship between these disorders and CD is not well established, some authors believe that the elevated concentration of NO could be useful in identifying the patients who may derive the greatest therapeutic benefit from GFD.[23]

It is interesting that patients on GFD in our study had also elevated NO serum levels despite significant decline in antibody levels. This may indicate that nitrosative stress in CD patients persists despite GFD, serological and clinical remission, and may be responsible for persistent histopathological changes. On the other hand, it may point to the difficulty in complete elimination of all sources of gluten in modern diet; perhaps the NO concentration is a more sensitive marker than TGA in the detection of trace amounts of gluten in diet.

The main antioxidant enzymes are SOD, GPx, and catalase. GPx is involved in elimination of lipid peroxides using GSH as a reducing factor.[24] Ståhlberg et al[25] reported a decreased expression of GPx in small intestinal mucosa in children with total villous atrophy. Stojiljković et al[26] showed a reduction of GSH levels and decreased GPx and GR activity in the peripheral blood of celiac children. In subsequent papers, the same authors reported that GPx activity in the small intestine was also significantly lower in children with untreated and silent CD than in controls, and they showed a positive correlation between GPx activity and both GR and GSH concentrations.[13,27]

To the best of our knowledge, no previous research has investigated serum GPx3 levels in adult celiac patients. In agreement with the above data, our results demonstrated a decreased activity of this antioxidant enzyme in the celiac group when compared with controls. A significant decrease in GPx3 expression was observed in patients with the active form of the disease, which means that antioxidant capacity in such patients may be reduced. In patients on GFD, the mean activity of GPx3 was slightly increased compared with the untreated group, but was lower than in controls. The pattern of the observed GPx3 activity in serum seems to be very similar to that reported by other authors in intestinal mucosa, suggesting that these changes may be systemic. The activity of GPx depends on the availability of GSH and selenium, the depletion of which was reported in celiac patients.[13,26,27] It appears that a reduction in GSH and selenium levels is followed by a decrease in GPx activity.

Dietary antioxidants such as vitamin E help maintain oxidative balance in a way similar to that of other antioxidants. Vitamin E ensures stability of biological membranes, thus protecting from harmful cellular effects of ROS, including the deleterious effects of lipid peroxidation.[28] Numerous disorders are related to changes in vitamin E levels, but it is unclear whether this is the result or the cause of the disease. There have been numerous reports on the insufficiency of fat-soluble vitamins in adult subjects with gluten enteropathy.[29,30] The plasma or serum concentrations of α-tocopherol exceeding 16.2 μmol/l are considered as sufficient; the levels ranging from 11.6 to 16.2 indicate low vitamin E levels, and the levels of less than 11.6 μmol/l suggest a deficiency. Recently, it has been proposed that the adequate plasma concentration of α-tocopherol to prevent neoplasm and cardiovascular disorders is more than 30 μmol/l.[31] In contrast to Hozyasz et al,[32] who reported that in untreated patients the levels of plasma tocopherol were significantly lower compared with those on GFD,[32] we showed a significantly decreased serum concentrations of vitamin E in both celiac groups regardless of compliance with diet. Noteworthy, the GFD did not increase the level of this vitamin. Our observations may indicate that oxidative imbalance persists despite the exclusion of gluten and suggests the need for additional supplementation of dietary antioxidants.

We explored, for the first time, the serum levels of UA as a nonenzymatic plasma antioxidant in adult patients with CD. Hyperuricemia is included in metabolic syndrome, and numerous

authors have reported a positive correlation between the prevalence of metabolic syndrome and increased UA concentrations.[33,34] In the same way, Dao et al[34] reported a high rate of metabolic syndrome among patients with gout. To our knowledge, there have been no studies reporting an increased incidence of gout in celiac patients.

In our study, we excluded patients with diabetes and chronic renal impairment, and none of the celiac patients had been diagnosed with gout or severe dyslipidemia. Hence, it does not seem that the observed increase in serum UA concentrations resulted from metabolic disorders or GFD.

UA is considered a marker of oxidative imbalance as well as an antioxidant with a protective feature.[35,36] Elevation of the serum UA concentration occurs as a physiologic response to increased OS.[37] It is possible that a high level of UA reflects the specific mechanisms for the prevention or correction of oxidative damage. Our results may indicate that higher levels of UA in celiac patients compared with controls are a consequence of increased OS, and that UA may function as an antioxidant in this case. The role of UA in disorders related to OS remains unclear. Glantzounis et al[38] reported that UA acts as an antioxidant in vivo. UA also functions as a prooxidant by enhancing the levels of free radicals and inducing endothelial injury, inflammatory process, abnormalities in NO concentrations, and atherosclerosis.[38] More studies are required to explain the role of UA in CD and to examine its function as a marker of OS as well as an antioxidant.

Similarly to α-tocopherol, bilirubin is an antioxidant that blocks vascular cell adhesion molecule-1 expression in vitro.[39] Significantly lower bilirubin levels were reported in severe asthma in an Australian study.[40] This may suggest that bilirubin and antioxidant vitamins affected the inadequate control of inflammation in patients with asthma. This observation is consistent with our result, suggesting an altered concentration of bilirubin as a result of OS. However, the role of bilirubin in oxidative imbalance in CD patients requires further research.

Ferritin protects against OS by chelation with free iron in conditions of excessive OS.[41] Data on OS and the antioxidant defense system in patients with sideropenic anemia are limited and debatable.[42-44] Akça et al[45] showed increased OS in pediatric patients with iron-deficiency anemia and reported its normalization following treatment.[45] Potaczek et al[46] showed that iron deficiency is associated with an increased rate of venous thromboembolism, and one of the postulated mechanism behind that finding was reduced antioxidant defense due to iron deficiency and reduced GPx activity.[47] Iron deficiency in celiac patients may have an additional effect on the severity of OS; therefore, an adequate treatment of this deficiency may be important to enhance antioxidant defenses.

In our study, vitamin D deficiency was observed in celiac patients despite the absence of classic clinical malabsorption syndrome. Inflammation may lead to vitamin D deficiency. It is likely that proinflammatory cytokines, such as tumor necrosis factor α, contribute to conversion of 25(OH)D to $1,25(OH)_2D$ in the intestine, hence lowering the serum concentration of 25(OH)D. In addition, $1,25(OH)_2D$ is involved in the inhibition of the production of proinflammatory cytokines by type-1 helper T cells, thus reducing inflammation.[48] This inverse relationship between the activity of CD and serum vitamin D levels was reported in our current study.

Cholecalciferol (vitamin D_3) and its active metabolite were found to be membrane antioxidants.[48] Their antioxidant properties are rather newly recognized and less well studied. Vitamin D_3 probably contributes to the stability of biological membranes and protects them from the products of lipid oxidation.[48] Vitamin D_3 exerts its antioxidant functions also by affecting the antioxidant enzymes.[49] Our study showed a decreased serum activity of GPx3 in the study groups with reduced serum vitamin D_3 levels. Inflammation with overexpressed tumor necrosis factor α may lead to reducing serum vitamin D_3 levels, and on the other hand, reduced concentrations of vitamin D_3 seem to be one of the causes leading to the impairment of antioxidant defense. This observation indicates that early diagnosis of vitamin D_3 deficiency is very important in patients with CD, particularly in those who do not comply with GFD. It seems to be important not only for bone metabolism but also for effective treatment of intestinal damage by reducing OS. This is especially important because, according to a large-scale Polish study,[50] only a very limited percentage of the urban population (9.1%) have adequate 25(OH)D levels, which is consistent with the European and American reports on the vitamin D_3 status. The effect of the vitamin D_3 status on OS in patients with CD requires further investigation.

A limitation of our study is that we did not assess the intestinal levels of GPx, NO, and vitamin E to compare the pattern of concomitant alterations in intestinal mucosa.

In conclusion, oxidative imbalance appears to be one of the main pathomechanisms of CD by affecting intestinal damage, the disease course, and perhaps extraintestinal disorders. The observed alterations in the serum concentrations of the above parameters of OS and antioxidants may suggest that these changes in oxidative imbalance are systemic and can contribute to extraintestinal manifestations. Our results indicate that OS persists even in treated patients, although to a lesser extent, and that GFD is only partially able to improve oxidative imbalance. The serum NO level seems to be a marker of the effectiveness of treatment, but further studies are necessary to clarify this issue and to elucidate the potential role of UA in CD as a marker of OS and its potential therapeutic role as an antioxidant. Considering that OS is involved in the molecular mechanisms of CD, the additional effect of such

antioxidants as vitamin E on oxidative imbalance may prove to be an effective adjuvant therapy, besides a rigorous GFD.

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Contribution statement AP-G and MZ-W conceived the idea of and designed the research; AP-G and MP-F conducted the literature search and study selection; MZ-W assessed the quality of the included studies; AP-G, AP-B, PP, PZ, and MK performed the research; PP and PZ analyzed the data; AP-G and MZ-W wrote the paper; PZ and TM revised the manuscript for final submission.

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